Applied and Environmental Microbiology, Dec. 2009, p. 7814–7821 Vol. 75, No. 24
0099-2240/09/$12.00 doi:10.1128/AEM.01333-09

Genetic Features of Resident Biofilms Determine Attachment of *Listeria monocytogenes*▽

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Received 8 June 2009/Accepted 7 October 2009

Planktonic *Listeria monocytogenes* cells in food-processing environments tend most frequently to adhere to solid surfaces. Under these conditions, they are likely to encounter resident biofilms rather than a raw solid surface. Although metabolic interactions between *L. monocytogenes* and resident microflora have been widely studied, little is known about the biofilm properties that influence the initial fixation of *L. monocytogenes* to the biofilm interface. To study these properties, we created a set of model resident *Lactococcus lactis* biofilms with various architectures, types of matrices, and individual cell surface properties. This was achieved using cell wall mutants that affect bacterial chain formation, exopolysaccharide (EPS) synthesis and surface hydrophobicity. The dynamics of the formation of these biofilm structures were analyzed in flow cell chambers using in situ time course confocal laser scanning microscopy imaging. All the *L. lactis* biofilms tested reduced the initial immobilization of *L. monocytogenes* compared to the glass substratum of the flow cell. Significant differences were seen in *L. monocytogenes* settlement as a function of the genetic background of resident lactococcal biofilm cells. In particular, biofilms of the *L. lactis* chain-forming mutant resulted in a marked increase in *L. monocytogenes* settlement, while biofilms of the EPS-secreting mutant efficiently prevented pathogen fixation. These results offer new insights into the role of resident biofilms in governing the settlement of pathogens on food chain surfaces and could be of relevance in the field of food safety controls.

Listeria monocytogenes is a food pathogen that has been implicated in numerous food-borne disease outbreaks (5, 58). This organism is found not only in food products but also on surfaces in food-processing plants (18). It is well documented that *L. monocytogenes* is able to adhere and form persistent biofilms on a variety of solid materials, such as stainless steel, glass, or polymers (18, 48, 51, 52). However, in food-manufacturing plants (and particularly in fermented-food-processing environments), it is most likely that the first contact between a pathogen and a surface will concern a resident microbial biofilm covering the solid surface (10, 35, 46). In this context, such a resident biofilm may be regarded as a "conditioning film" that modifies the topographic and physicochemical characteristics of the surface and hence the adhesion capability of planktonic microorganisms coming into contact with this substratum (6).

Once the pathogens are immobilized on the surface, interactions between the pathogens and their environment (physiological interactions with resident flora, nutrient availability, pH, water activity, temperature, and cleaning and disinfection procedures) govern the long-term settlement and persistence of the pathogens on the surface. Various studies have demonstrated the inhibition of *L. monocytogenes* development by natural "protective" biofilms (10, 66). Competition for nutrients has been demonstrated as a major mechanism underlying the inhibition of pathogen development (25, 27). The production of antimicrobial agents (bacteriocins, acids, and hydrogen peroxide) has also been reported as being of importance to such interactions (13, 20, 36). For example, *Lactococcus lactis* has been described as being exceptionally efficient in controlling the development of *L. monocytogenes* on food-processing surfaces by means of competitive exclusion (66) or bacteriocin production (35). It has been reported that treating a surface with a bacterial polysaccharide prevented the adhesion of different nosocomial pathogens (60). Furthermore, alginate-overexpressing *Pseudomonas aeruginosa* biofilms reduced the retention of *Cryptosporidium parvum* oocysts (54). Other recent studies have shown that the composition and quantity of specific exopolysaccharides (EPS) in *Pseudomonas* biofilms can inhibit the fixation of *Escherichia coli* or *Erwinia chrysanthemi* planktonic cells in porous media (37, 38).

The present study investigated those properties of resident biofilms that could affect the settlement of *L. monocytogenes*. *L. lactis* was used as a model resident biofilm strain, as this is widely used in dairy fermentations and its cell wall properties have been the subject of considerable study (22, 23). Cell wall mutants of *L. lactis* MG1363 were used to create a set of model biofilms that differed in terms of their architecture, EPS synthesis, and cell surface hydrophobicity. These biofilms were used to evaluate the attachment of fluorescent inert polystyrene microbeads and of two reference strains of *L. monocytogenes* (LO28 and EGDe) using in situ confocal fluorescence imaging.

MATERIALS AND METHODS

Biological materials. The bacterial strains used during this study are listed in Table 1. *L. lactis* strain MG1363 (as a wild type [WT]) and the following widely described derivatives were used, as they are known to affect cell surface and

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▽ Published ahead of print on 16 October 2009.

TABLE 1. Bacterial strains and plasmids used during the study

Strain or plasmid	Properties[a]	Reference
Lactococcus lactis strains		
MG1363	WT strain, plasmid-free and prophage cured	21
MG1363 Δ*acmA* mutant	Inactivation of *N*-acetyl glucosaminidase, long-chain formation	9
MG1363/pNZ4030 (EPS^+)	Em^r	61
MG1363/pGKV552 ($PrtP^+$)	Em^r, active PrtP protease expression	33
Listeria monocytogenes strains		
LO28	WT, serovar 1/2c	43
EGDe	WT, serovar 1/2a	44
EGDe::pNF8	Em^r, derivative of *L. monocytogenes* EGDe harboring pNF8	24
LO28::pNF8	Em^r, derivative of *L. monocytogenes* LO28 harboring pNF8	19
Plasmids		
pGHost8	Tet^r, vector plasmid	41
pRV85	Em^r, 6.2-kb derivative of pGHost5 carrying a 1,014-bp pldhL-GFP gene fragment	26
pGHost8-gfp2	Tet^r, derivative of pGHost8 carrying a 1,014-bp pldhL-GFP gene cassette obtained after restriction by EcoRI	This work
pNF8	Em^r, derivative of pAT18 carrying pdlt and GFP-mut1 fragments	19
pGKV552	Contains the replication origin of pWV01 and the cloned *prtPI* gene	29
pNZ4030	EPS-producing plasmid carrying erythromycin and chloramphenicol resistance genes	61

[a] Em^r, erythromycin resistant; Tet^r, tetracycline resistant.

adhesive properties: NZ4030 (here called EPS^+), MG1363/pGKV552 ($PrtP^+$) and MG1363 Δ*acmA*.

The EPS^+ strain carries plasmid pNZ4030, encoding a cluster of 14 genes that results in EPS synthesis and secretion (61). This strain was shown to exhibit a poor ability to adhere to different solid surfaces (S. Kulakauskas, data not shown). The $PrtP^+$ strain carries the plasmid-encoded, cell wall-anchored proteinase PrtPI and has been shown to increase cell surface hydrophobicity and adhesion to solid surfaces (30). The peptidoglycan hydrolase-negative Δ*acmA* strain (42) has defective cell separation and thus forms long chains. This strain also expresses both autolysis-negative (42) and poor-adhesion (32) phenotypes. Green fluorescent protein (GFP) expression in lactococcal strains was achieved by introducing a pGHost8-gfp2 plasmid containing both a tetracycline resistance cassette and a GFP cassette under control of an *ldhL* promoter. A 1,014-bp fragment including the GFP gene under the control of the *ldhL* promoter was obtained after EcoRI digestion of pRV85. This fragment was ligated to an EcoRI digest of pGHost8 (41). The resulting pGHost8-gfp2 plasmid was selected as a tetracycline-resistant clone in *L. lactis*.

Growth characteristics. *L. lactis* strains were grown in M17 medium (Oxoid, France) supplemented with 0.5% glucose. Bacteria were subcultured twice at 30°C and then cultivated overnight at 30°C. The *L. monocytogenes* EGDe and LO28 strains were subcultured twice and grown overnight in tryptic soy broth medium at 30°C under agitation. When required, erythromycin (Fluka, France) and/or tetracycline (Sigma, France) was added at a final concentration of 5 μg/ml.

Bacterial cell surface hydrophobicity and polarity. Bacterial cell surface hydrophobicity and polarity were estimated using the microbial adhesion to solvents (MATS) method. This is a partitioning test based on comparing the affinities of microbial cells with monopolar (e.g., chloroform) and apolar (e.g., hexadecane) solvents, both of which express identical van der Waals surface tension components (3). Experimentally, 2.4 ml of a bacterial suspension in 150 mM NaCl was vortexed for 60 s with 0.4 ml of the solvent under investigation (chloroform or hexadecane [Sigma]). The mixture was allowed to stand for 15 min to ensure complete separation of the two phases before a sample (1 ml) was carefully removed from the aqueous phase and its optical density measured at 400 nm. The percentage of cells present in each solvent was subsequently calculated using the equation $\%\ \text{affinity} = 100 \times [1 - (A/A_0)]$, where A_0 is the absorbance of the bacterial suspension before mixing, measured at 400 nm, and A is the absorbance after mixing. Each experiment was performed in triplicate using independently grown cultures.

Bacterial cell surface electrical charge. The bacterial cell surface electrical charge was evaluated at pH 6 in 1.5 mM NaCl by electrophoretic mobility using a laser zetameter (CAD Instrumentation, France), as described previously (30). Each experiment was performed in triplicate using independently grown cultures.

Biofilm growth. Biofilms were cultivated at 25°C in disposable three-channel flow cells (Stovall, Bioblock, France) with individual channel dimensions of 1 by 4 by 40 mm and a sealed glass coverslip substratum. To inoculate the flow cells, 1 ml of an exponential-phase culture adjusted to an optical density at 600 of 0.01 was injected into each channel using a sterile syringe. Static conditions (no flow) were maintained for 1 h after inoculation to allow initial bacterial adhesion (time zero). After this period, the flow was resumed at 2 ml/h using a Watson-Marlow 205S peristaltic pump (Watson-Marlow Ltd., Falmouth, England), and the three-dimensional (3D) structure of biofilms was subsequently analyzed using confocal microscopy at 4 h and 24 h after flux activation. In order to limit acidic pH interference with GFP expression in *L. lactis* biofilms, the pH of the flow medium was stabilized at pH 7 by the addition of 0.19 M MOPS (morpholinepropanesulfonic acid) (Sigma, France) and NaOH.

In situ time course CLSM of *Lactococcus* biofilms. Horizontal plane images of the biofilms were acquired using a Leica SP2 AOBS confocal laser scanning microscope (CLSM) (Leica Microsystems, France) at the MIMA2 microscopy platform (http://voxel.jouy.inra.fr/mima2). The excitation wavelength used for GFP was 488 nm, and emitted fluorescence was recorded within the range of 500 to 550 nm. Images were collected through a 63× Leica oil immersion objective (numerical aperture = 1.4) with a z step of 1 μm.

3D projections were performed with IMARIS software (Bitplane, Zürich, Switzerland). The structural quantification of biofilms (biovolume and thickness) was performed using the PHLIP Matlab program developed by J. Xavier (http://phlip.sourceforge.net/phlip-ml).

Extracellular DNA staining. A 1 μM solution of 7-hydroxy-9H-(1,3-dichloro-9.9-dimethylacridin-2-one) (DDAO) was injected into each flow cell channel in order to fluorescently stain extracellular DNA in the biofilms. The dye was allow to react with DNA for 15 min in the dark before the fluorescent profile was recorded for different biofilm thicknesses (excitation, 633 nm; fluorescence emission collected within the range of 640 to 700 nm).

Metabolic activity profiles. The metabolic activity of bacteria within the biofilm was evaluated as a function of intracellular esterase activity by staining with ChemChrom V6 (AES-Chemunex, Inc., Princeton, NJ). When degraded by intracellular esterase, this cell-permeative and reducible substrate releases impermeative fluorescent products into the cell cytoplasm. A solution of 1 μM was injected into each flow channel and left in the dark for 30 min at 30°C. Bacterial esterase activity was recorded at different biofilm thicknesses (excitation, 488 nm; fluorescence emission collected within the range of 500 to 600 nm).

Ex situ SEM of *Lactococcus* biofilms. For scanning electron microscopy (SEM) observations, *L. lactis* biofilms were cultivated, chemically fixated, and dehydrated in single-channel BST FC81 flow cells (Biosurface Technologies Corporation, Bozeman, MT; channel dimensions, 1.6 by 12.7 by 47.5 mm). These reusable flow cells are not sealed, so that the glass substratum can be recovered with the attached biofilms for ex situ procedures such as SEM observations.

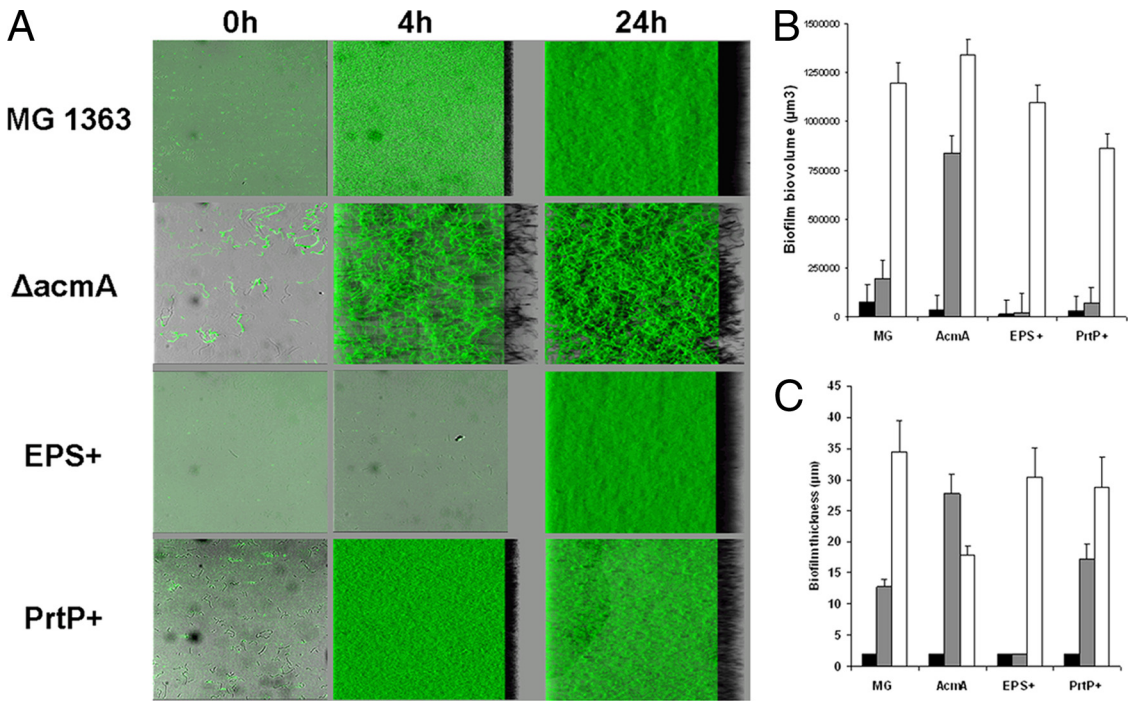

FIG. 1. (A) Time-lapse CLSM of GFP-tagged WT *L. lactis* and its cell wall mutants. The structural development of biofilms was followed using time lapse CLSM. The side view projections shown here were acquired after 0 h, 4 h, and 24 h of biofilm development. (B) Biovolume analyzed by PHLIP on *L. lactis* WT, Δ*acmA*, EPS$^+$, and PrtP$^+$ biofilms after growth for 0 h (black bars), 4 h (gray bars), and 24 h (white bars). (C) Thickness analyzed by PHLIP on *L. lactis* WT, Δ*acmA*, EPS$^+$, and PrtP$^+$ biofilms after growth for 0 h (black bars), 4 h (gray bars), and 24 h (white bars). Error bars indicate standard errors.

Biofilms grown for 24 h, as described above, were rinsed with a 150 mM NaCl solution to remove planktonic cells and then fixated for 4 h in a solution containing 2.5% glutaraldehyde, 0.1 M sodium cacodylate, and 0.075% ruthenium red at pH 7.4. After three washes in 0.1 M sodium cacodylate buffer, the biofilms were dehydrated in ethanol and placed in 50% and then 100% hexamethyl disilizane. The samples were dried in air and then coated with palladium for 210 s at 800 V and 10 mA. All the chemicals were obtained from Agar Scientific, Ltd. (England). Ex situ high-magnification imaging of the biofilms was performed under a S-4500 Hitachi SEM (Hitachi, Japan) at the MIMA2 microscopy platform.

Adhesion of polystyrene beads to *Lactococcus lactis* biofilms. Before the experiments with GFP-tagged *Listeria* cells, the adhesion of inert anionic polystyrene 2-μm microbeads to the surface of an *L. lactis* biofilm was realized as described previously (6). Anionic polystyrene microbeads were used to mimic the behavior of single bacterial cells in biofilm studies. These are especially useful to study the role of physicochemically defined parameters in adhesion (6). Experimentally, 300 μl of a fluorescent, carboxylate-modified latex bead solution at 2.5% (L4530 Sigma) was added to 30 ml of 150 mM NaCl. After thorough mixing, the beads were washed three times in 150 mM NaCl. The beads were then dispersed by 10 min of sonication at 25°C prior to utilization. The suspension of fluorescent polystyrene beads was then injected into the flow cell channels containing 24-h biofilms using a Watson-Marlow 205S peristaltic pump (Watson-Marlow Ltd., Falmouth, England). Once the bead solution had filled the flow channels, the flow was stopped for 1 hour to allow static bead adhesion to the *L. lactis* biofilms. After this period, a solution of 150 mM NaCl was pumped through the flow cell channels for 30 min to eliminate nonadherent beads. Adherent fluorescent beads were quantified throughout the biofilm volume using 3D confocal imaging and image analysis. Fluorescent beads were irradiated at an excitation wavelength of 543 nm with an HeNe laser through a 63× objective (numerical aperture = 1.4), and the resulting fluorescence was collected within the range of 590 to 625 nm. Images were acquired on the entire thickness of the biofilms with a step of 1 μm. Adherent polystyrene beads were quantified using ImageTool (University of Texas Health Science Center at San Antonio, TX).

Adhesion of *L. monocytogenes* to *L. lactis* biofilms. An overnight culture of 10 ml 10^8 GFP-tagged *L. monocytogenes* LO28 and EGDe cells was washed in 150 mM NaCl using a series of three centrifugations (7,000 × *g*, 4°C 10 min). An *L. monocytogenes* suspension was then pumped into the flow cell channels containing 24-h biofilms using a Watson-Marlow 205S peristaltic pump (Watson-Marlow Ltd., Falmouth, England). Once the suspensions of *L. monocytogenes* had filled the flow cell channels, the flow was stopped for 1 hour to allow bacterial adhesion to the biofilms. After this period, a solution of 150 mM NaCl was pumped through the flow cell channels for 30 min to eliminate nonadherent bacteria. Fluorescent adherent *L. monocytogenes* cells were quantified throughout the biofilm volume using 3D confocal imaging (with the GFP settings described above), and the biovolume of *L. monocytogenes* was estimated with the PHLIP Matlab program.

RESULTS

Dynamics of *L. lactis* biofilm formation. To evaluate the dynamics of lactococcal biofilm structure and formation, the growth of GFP-labeled *L. lactis* cells was monitored in flow chambers.

The dynamics of *L. lactis* biofilm structure and formation in flow cells were followed and quantified using time course CLSM. Figure 1A shows representative 3D projections of *L. lactis* MG 1363 and its cell wall mutants at 0 h, 4 h, and 24 h after activation of the medium flow. Such images were used to evaluate the structural quantification parameters biovolume and thickness (Fig. 1B and C). We also evaluated the structure of a 24-h biofilm using high-magnification SEM (Fig. 2). The results showed that biofilms obtained with the WT strain displayed a rapid coverage of the substratum and development of a dense and uniform mushroom-less structure that reached a thickness of ~35 μm after 24 h (Fig. 1C). The PrtP$^+$ strain presented a structural architecture similar to that of the WT ($P > 0.05$ for the biovolume parameter). In comparison to the

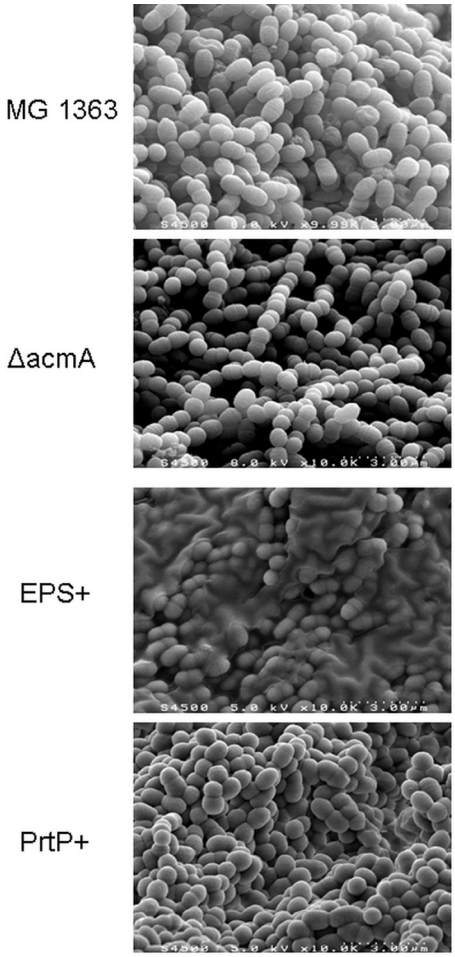

FIG. 2. SEM images of *L. lactis* WT, Δ*acmA*, EPS$^+$, and PrtP$^+$ strains after 24 h of growth at 25°C in a flow cell.

WT, markedly slower biofilm formation was observed for the EPS-producing strain (no biofilm initiation 4 h after flow activation; $P < 0.05$ for the biovolume parameter in comparison with WT [Fig. 1B]). Identical growth rates were obtained for the WT and the EPS-producing strain (data not shown). This indicates that the bacterial growth rate is not the reason for the slow *L. lactis* EPS$^+$ surface colonization. This initial delay in biofilm formation could be attributed to the poor adhesion efficiency and slippery properties of EPS-producing cells under dynamic flow conditions. Nevertheless, this strain had formed a tight and compact biofilm after 24 h. SEM revealed abundant extracellular material covering the surface of the EPS$^+$ biofilm. This feature was attributed to the production of lactococcal EPS (Fig. 2).

The Δ*acmA* strain formed a biofilm with an apparently porous structure (Fig. 1A). This type of biofilm structure, as well as a high bacterial biovolume and thickness after 4 h, could be attributed to the long bacterial chain formation by the Δ*acmA* mutant (9).

Surface physicochemical properties of bacterial cells and latex beads. During our experiments, we used 2-μm fluorescent carboxylate polystyrene beads to mimic the adhesion of *L. monocytogenes* to lactococcal biofilms. In order to compare the surface properties of these beads with those of *Listeria* cells, their surface charge was measured using electrophoretic mobility measurements and their hydrophobicity and polarity using the MATS test (see Materials and Methods).

Both fluorescent carboxylate polystyrene beads and *L. monocytogenes* cells were electronegative, with the beads being more negatively charged (electrophoretic mobility at pH 6 within the range of -2.4×10^{-8} to -6.0×10^{-8} m^2 V^{-2} s^{-1}) (Table 2). Beads and *Listeria* cells showed similarly weak Lewis base characteristics (higher affinity to chloroform than to hexadecane), and both were slightly hydrophobic (affinity to hexadecane within the range of 65% to 73%), as presented in Table 2. Hence, under our experimental conditions, the carboxylate-modified beads exhibited surface physicochemical properties similar to those of the selected *Listeria monocytogenes* strains.

The same tests were employed to evaluate the physicochemical surface properties of *L. lactis* cell wall mutants. As described previously (30), the surface of strain MG1363 was hydrophilic (no affinity to hexadecane). The presence of anchored PrtPI proteinase in its active form markedly reversed this character to hydrophobicity (affinity to hexadecane of >88%) (Table 2). the Δ*acmA* and EPS$^+$ mutants were found to display a weak affinity for hexadecane that was similar to that of the WT. However, the Δ*acmA* mutant exhibited a stronger affinity for chloroform, suggesting the Lewis base character of this mutant.

Electrophoretic mobility measurements revealed a global electronegative charge for WT *L. lactis* and its cell wall mutants at pH 6 (-2.5×10^{-8} to -3.5×10^{-8} m^2 V^{-2} s^{-1}) (Table 2). However, the electronegativity of cells harboring an anchored PrtPI proteinase or overexpressing EPS was significantly altered ($P < 0.05$).

Immobilization of *Listeria monocytogenes* and polystyrene microbeads on *Lactococcus lactis* biofilms. In order to mimic the settlement of planktonic bacteria on WT *L. lactis* and its cell wall mutants using biofilms as model substrata, inert microspheres were first employed as a model. A clear reduction in bead adhesion to the *L. lactis* WT biofilm was observed, compared with that to a sterile glass surface (Fig. 3A). Bead adhesion was not affected in the biofilm-forming PrtP$^+$ strain compared to WT *L. lactis* ($P > 0.05$).

TABLE 2. Summary of physicochemical measurements for the *L. lactis* cell wall mutants tested

Strain	Affinity (%)[a] to:		Electrophoretic mobility (10^{-8} m^2 V^{-2} s^{-1}) at pH 6[b]
	Chloroform	Hexadecane	
L. lactis MG1363			
WT	0	4	−3.2
Δ*acmA*	45	4	−3.5
EPS$^+$	0	0	−2.8
PrtP$^+$	80	88	−2.5
L. monocytogenes			
LO28	92	65	−2.6
EGDe	91	65	−2.4
Polystyrene microbeads	98	73	−6.0

[a] The standard deviations were lower than 8% of the mean values.
[b] The standard deviations were lower than 13% of the mean values.

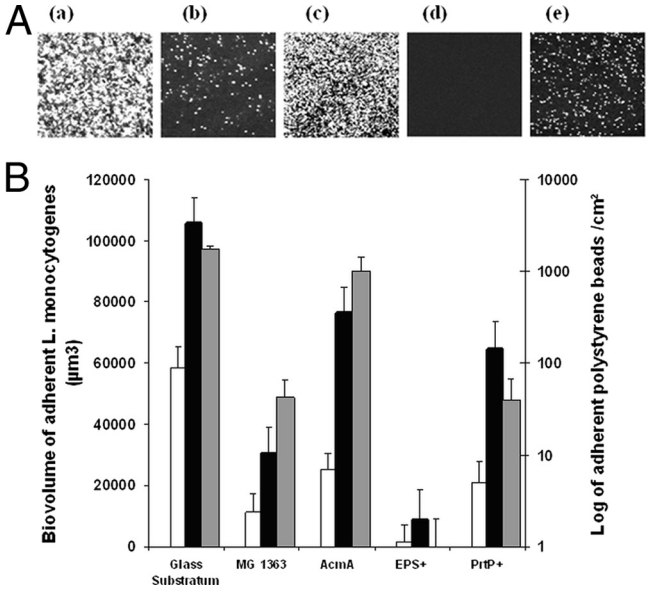

FIG. 3. (A) 2D CLSM projections of carboxylate latex beads adhered to a glass substratum (a) and 24-hour biofilms of *L. lactis* WT (b), Δ*acmA* (c), EPS$^+$ (d), and PrtP$^+$ (e) strains. (B) Adhesion of *L. monocytogenes* EGDe (white bars) and *L. monocytogenes* LO28 (black bars) to a glass substratum and to *L. lactis* biofilms after 1 h at 25°C. Adhesion, represented as biovolume, was analyzed using PHLIP. Gray bars indicate the adhesion of polystyrene latex beads to a glass substratum and to *L. lactis* biofilms after 1 h at 25°C (right axis). Error bars represent standard errors.

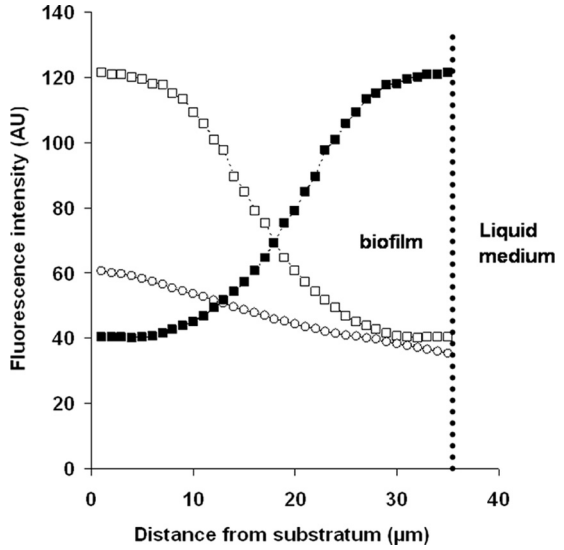

FIG. 4. Fluorescence intensity profile of the red DNA intercalating agent DDAO throughout the biofilm thickness in *L. lactis* WT (□) and Δ*acmA* (○) 24-h biofilms and ChemChrom V6 profile for the biofilm obtained from the WT (■). The glass substratum is represented at 0 μm.

The most marked effect was observed with respect to EPS$^+$ biofilms: bead fixation to the biofilm was almost totally inhibited (38.6 times less adhesion than for the WT; $P < 0.05$). In contrast, "porous" biofilms obtained from the Δ*acmA* strain retained 24.0 times as many beads as the WT ($P < 0.05$). The Δ*acmA* biofilm had another notable and distinctive feature: beads were detected throughout the Δ*acmA* biofilm volume and not only at the biofilm-flow medium interface (as was the case for other strains).

The pattern of attachment of live GFP-tagged *L. monocytogenes* EGDe and LO28 cells to lactococcal biofilms was found to be similar to that of latex beads. In particular, we found that (i) adhesion was significantly inhibited by all the *L. lactis* biofilms compared to adhesion to a glass surface ($P < 0.05$) (Fig. 3B); (ii) *L. monocytogenes* adhesion was almost prevented on EPS$^+$ biofilms ($P < 0.05$ for both the LO28 and EGDe strains) compared with the *L. lactis* WT strain; (iii) *L. monocytogenes* adhesion on a Δ*acmA* mutant biofilm was at its maximum ($P < 0.05$ for both the LO28 and EGDe strains); (iv) *Listeria* cells were distributed throughout the Δ*acmA* biofilm volume; and (v) *Listeria* adhesion to the PrtP$^+$ biofilm was comparable to the results obtained with the WT biofilm, except that *Listeria monocytogenes* LO28 was 2.2 times more adherent than on the WT ($P < 0.05$).

Differences were also observed during these experiments between the two *Listeria* strains tested: *L. monocytogenes* LO28 always adhered more to biofilm surfaces than EGDe ($P < 0.05$, excluding EPS$^+$ biofilms, where the adhesion of both strains was almost totally inhibited).

Profiles of cellular metabolic activity and extracellular DNA content in biofilm matrices. In biofilm-forming bacteria, chromosomal DNA serves not only as a genetic material but also as a structural component of the biofilm (49, 50, 65). There is controversy in the literature concerning the role of autolysis as it affects DNA release in the matrix (64). Because the Δ*acmA* mutant is deficient in the principal lactococcal autolysin and therefore deficient in bacterial autolysis (9), it constituted a good model to evaluate the effect of lysis on DNA release in the matrix. For this reason, the extracellular DNA content was evaluated in WT and Δ*acmA* mutant biofilms. The DNA content was determined by specific staining using the cell-impermeative DDAO red fluorescent dye (65). Profiles of fluorescence intensity within the WT and Δ*acmA* biofilms are presented in Fig. 4. Biofilms obtained using the WT strain displayed bright red fluorescence that decreased from the solid surface toward the medium interface, thus suggesting the presence of a DNA concentration gradient within the biofilm, with a higher concentration at its base. In comparison with the WT, the extracellular DNA content in the deeper layers of the Δ*acmA* biofilm was very low (Fig. 4). Because the Δ*acmA* mutant is defective in the major cell autolysin that is responsible for cell lysis, this could suggest that extracellular DNA is mostly a product of cell lysis in the WT strain. The presence of high DNA concentrations in deeper layers could be related to more efficient autolysis. This reasoning corresponds to the findings that lactococcal cells lyse more efficiently in stationary growth phase (9), the conditions characteristic of deeper biofilm layers (56).

In order to evaluate the metabolic activity of bacteria in the biofilm, esterase activity profiles were determined using the green fluorescent ChemChrom V6 dye (Fig. 4). These measurements indicate a higher esterase activity near the fluid interface, in close proximity to the fresh nutrient.

DISCUSSION

Listeria monocytogenes is able to adhere to a large number of living surfaces (plant tissue [53] or human epithelial cells [11]) or abiotic surfaces (stainless steel [40, 52, 62] or polymers [4, 14]). The surface adhesion of *L. monocytogenes* is governed by the properties of both the cell surface and the substratum. The most influential cellular factors have been reported to be hydrophobicity (7, 15, 40), electric charge (1, 8), flagella (62), and specific bacterium-host cell interactions (12). However, no published reports have revealed the aspects of resident biofilms that are involved in their interactions with planktonic bacterial pathogens. We approached this question by testing the attachment of two strains of *Listeria monocytogenes* with different biofilm phenotypes to the surface of customized *Lactococcus lactis* biofilms.

The development of *L. lactis* WT and cell wall mutant biofilms was monitored at 0, 4, and 24 h. In contrast to the previously observed weak biofilm development under static conditions (28, 42, 66), the WT strain (*L. lactis* MG1363) formed thick, flat, and structurally homogenous biofilms under flow conditions. Fluorescent staining demonstrated the existence of a horizontal stratification within this biofilm: metabolically active cells were present in the upper layers (in direct contact with the growth medium), and larger quantities of extracellular DNA were found in the base of the biofilm. We expected autolysis to be important for biofilm formation because released DNA can act as a "cementing" agent for biofilm cohesion (49, 50, 65). The development of a biofilm using a strain deficient in the major cell wall autolysin, the Δ*acmA* strain, dramatically reduced the extracellular DNA content in the biofilm. Because AcmA is the main lactococcal autolysin (9), the low DNA content was attributed to diminished cell lysis in the Δ*acmA* mutant. In the WT strain, cell lysis tended to occur more efficiently in deeper layers of the biofilm, where cells were less exposed to fresh nutrients and consequently were less metabolically active. Therefore, bacteria situated in the deeper layers of the biofilm would be expected to be similar to stationary-phase planktonic cells (56), conditions which favor lactococcal cell lysis by AcmA (9).

The lower DNA content was probably the reason for the observed "loose" structure of the Δ*acmA* biofilm, resulting in the deep penetration of fluorescent microspheres. We suggest that cell cohesion in the biofilm, and consequent resistance to flow were mainly the result of long-chain formation and 3D entanglement. Chain formation has been described in other bacterial models as a means of developing cohesive 3D biofilms without extensive matrix formation (51). A previous study conducted under static conditions had also indicated that the Δ*acmA* mutant was a poor producer of biofilms compared to the WT (32, 42). As observed in other bacterial models (50, 65), extracellular DNA is thus one of the principal components of the *L. lactis* biofilm matrix. The presence of this extracellular DNA could be associated with the presence of other cytoplasmic or cell wall components of dead bacterial cells, such as lipids, proteins, teichoic acids, and polysaccharides (49, 50, 65). It has recently been hypothesized that cell death and lysis of a subpopulation may be essential to intercellular cohesion and biofilm strength (2, 50, 55, 63).

The dynamics of the *L. lactis* biofilm structure were also altered by the abundant synthesis of EPS. In this case, biofilm development was markedly delayed compared to that for the WT (very weak initial adhesion at 4 h after the cell injection). Nevertheless, the biofilm volume reached that of the WT during the later stages of development (there were no differences in biovolume after 24 h of growth). This delayed biofilm formation could be attributed to the poor adhesive ability of EPS-producing cells under flow conditions, as was shown previously (39). The poor adhesive ability of EPS-producing cells could be explained by the shear forces caused by the laminar flow regimen prevailing in flow cells. Moreover, SEM images of mature EPS^+ biofilm clearly showed the existence of abundant exocellular substances. This material was not observed on WT biofilms, so it could thus be attributed to EPS production.

Alterations to cell surface hydrophobicity and surface charge due to the presence of the anchored PrtPI protease did not reveal any structural dynamic in the biofilm compared to the WT. This suggests that although anchored protease PrtPI is important to adhesion under static conditions (30), it is not essential in terms of the growth and 3D structuring of *L. lactis* biofilms under flow conditions.

The adhesion of *Listeria* strains LO28 and EGDe and inert polystyrene microbeads was assessed on lactococcal biofilms. The use of abiotic beads enabled an evaluation of physicochemical adhesion properties by comparison with live *Listeria* cells, where adhesion might also be influenced by biological factors, e.g., specific recognition processes.

A marked decrease was observed in the adhesion of both *Listeria* cells and beads on lactococcal WT biofilms, compared with the inert glass substratum. This result was unexpected, as the biofilm surface was far rougher than that of glass, and this might favor adhesion. The physicochemical properties of lactococcal cells could hardly explain such a reduction in adhesion. For example, the hydrophobicities of the lactococcal strains used during this study differed markedly (only 4% hexadecane affinity for MG1363 but 88% for the $PrtP^+$ strain). The physicochemical properties of biofilm surfaces may have differed from those of planktonic cells, thus contributed to repelling *Listeria* cells (6). However, we attributed the reduction in planktonic cell adhesion to the biofilm to a phenomenon of continuous erosion at the biofilm-liquid interface (45). The lactococcal biofilms grown during our experiments reached a plateau (with biofilm thickness stabilizing at a level of 35 μm after approximately 24 h of growth), which might indicate the presence of an equilibrium between biofilm growth and detachment. A slight increase of CFU/ml in biofilm effluent between 24 and 48 h of incubation, suggests that cell detachment at the liquid-biofilm interface actually occurs (data not shown). It is therefore likely that adhered beads or *Listeria* cells on the biofilm surface would detach along with cells and aggregates of the biofilm and separate from the biofilm interface.

This explanation is in agreement with the increase in immobilized entities (pathogenic *Listeria* cells or microbeads) within the porous biofilms obtained using the chain-forming Δ*acmA* mutant. We could expect that when *Listeria* cells or beads entered deeper layers of the resident biofilm, they would be spared from such detachment. This reasoning would explain the increased presence of *Listeria* cells in Δ*acmA* biofilms. These observations were also in line with those of other studies of biofilms which demonstrated that the presence of pores and

voids in biofilms facilitated the capture and retention of colloidal particles (16, 17, 47). This highlights the possibility that "porous," Δ*acmA*-like resident biofilm structures could be favorable to "pathogen entrapment" and contribute to the danger of microbiological contamination of surfaces in the food chain.

In the case of both microbeads and *Listeria* cells, a dramatic decrease was observed in adhesion to biofilms obtained using EPS-producing cells of *L. lactis* compared to the WT. These observations were consistent with other findings that indicated a low retention of *Cryptosporidium parvum* oocysts on alginate-overexpressing *Pseudomonas aeruginosa* biofilms (54) and a reduction in the adhesion of nosocomial pathogens on a surface treated with a bacterial polysaccharide (60).

The EPS of the strain used during the present study has been reported to consist of pentasaccharide repeating units containing rhamnose, galactose, glucose, and phosphate at a ratio of 1:2:2:1 (61). The presence of a phosphate group in the repeating units generates anionic properties of the molecule at a physiological pH (59). The antiadhesive property of this biofilm could be linked to an abundance of negative charges which are highly repellent toward both anionic *Listeria* cells and microspheres. This effect may also be linked to the rheological or physical properties of EPS (31). Moreover, the antifouling properties of the EPS biofilm surface (in terms of the attachment of bacteria or beads) could be also explained by detachment forces; in addition to the detachment of biofilm fragments, the EPS material itself, together with immobilized bacteria, could also be detached. Compared to 24-h *L. lactis* WT biofilms, EPS biofilms were shown to have higher numbers of detached cells during their 24-h formation (data not shown). Therefore, our results suggest that the EPS matrix, in addition to its role of stabilizing and protecting the biofilm, may prevent the fixation of planktonic entities.

The molecular origin of the difference in attachment between the two strains remains unknown. We tested without success the hypothesis of differences in their surface physicochemical properties. From other published papers, it appears that the presence of functional flagella is important in *L. monocytogenes* attachment to inert surfaces and biofilm formation. Their effects can be opposite depending on the experimental conditions, as they appeared to be beneficial for attachment under static conditions (34, 62) and detrimental to attachment in flow cell reactors (57). Hence, a difference in the number of flagella or in their activity could explain the difference in attachment observed between the two tested strains.

In addition, it can be noted that the residual adherent cells of *L. monocytogenes* on the biofilm surface exhibited poor multiplication compared to those on the control glass surface (data not shown), indicating that *L. lactis* biofilms interfere not only with pathogen attachment but also with pathogen physiology.

In conclusion, we created a set of model *L. lactis* resident biofilms with different architectures, porosities, types of matrices, and individual cell surface properties. These biofilms were then used to investigate the factors governing the initial immobilization of *L. monocytogenes* and microbeads on biofilm surfaces. The results indicate that (i) the adhesion of planktonic cells to the surface is decreased by the presence of a biofilm, (ii) this adhesion is almost prevented when EPS are produced by biofilm-forming cells, and (iii) adhesion is increased by biofilms with a porous structure that are formed by chain-making strains. These results may be important to the control of surfaces where the presence of porous biofilms could be a threat because of pathogen entrapment. The presence of "antifouling" EPS on a biofilm surface could constitute a novel tool (together with physiological interference) for the development of "protective" biofilms for use in agricultural, food, and health environments.

ACKNOWLEDGMENTS

We thank H. Bièrne, Institut Pasteur, Paris, for supplying GFP-labeled *Listeria monocytogenes* and Victoria Hawken for English revision.

Olivier Habimana was the recipient of a fellowship from Labhealth under Marie Curie contract MEST-CT-2004-514428. We also thank the Département de l'Essonnes for its financial support of the confocal microscope platform (ASTRE no. A02137).

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